CHAPTER 5

Nestin, c-met, and c-kit expression in differentiating neonatal beta-cells
ABSTRACT

Objective: Nestin, c-met, and c-kit, positive cells are found in the developing or regenerating pancreas and less in the adult pancreas in homeostasis. However, little is known about the characteristics of these cells.

Research Design and Methods: We investigated the expression of genes involved in pancreas development (c-met, c-kit, nestin, Beta2, Islet-1, Pax6, Pax4, Pdx-1, ngn3, Glut-2, insulin, glucagon and amylase). Next we performed immunofluorescent staining of nestin, c-met, and c-kit with proteins associated with the early (islet-1), mid- (ngn3), or late (Pax 4) stage of beta-cells differentiation. We also studied the differential capacity of these cells into endothelial (CD31) and nerve cells (β3-tubulin).

Results: Nestin is expressed by beta-cells in the early and mid-stage of differentiation and is absent on beta-cells at the late or end stage of differentiation. C-met and c-kit are receptors that are found on all stages of differentiation of beta-cells. Furthermore, in the islets of Langerhans, nestin, c-met and c-kit positive cells also express pancreatic polypeptide, somatostatin, CD31 and β3-tubulin.

Conclusions: In the pancreas heterogenous population of nestin, c-met and c-kit positive cells exist. These cells are indeed precursors for beta-cells; however a portion also contributes to vessel and nerve formation.

INTRODUCTION

The neonatal rat pancreas has the ability to form a complete adult pancreas in a period of not more than three weeks [1,2]. This unique ability of the neonatal pancreas is under the guidance of a number of cell types. Among these cells types are a population of cells expressing nestin [3], the receptor for hepatocyte growth factor (HGF); c-met [4,5], and the receptor for the stem cell growth factor (c-kit) [1,2,4-6]. These proteins and receptors are found in the developing or regenerating pancreas and less in the adult pancreas in homeostasis [1-6]. Cells expressing these proteins are of interest for understanding the growth mechanisms of the pancreas and may even have therapeutic consequences as they may serve as target for induction of regeneration of the pancreas such as in some forms of type II Diabetes.
Nestin, c-met, and c-kit expression in differentiating neonatal beta-cells

Nestin expression has been mainly associated with beta-cell formation. It has been described that nestin positive cells differentiate in vitro into endocrine cells that produce insulin, glucagon, glucagon like peptide-1 (GLP-1), and the transcription factor Pdx-1 [3]. A group of these nestin positive cells carry the c-met receptor and have been associated with pancreatic development [5,7,8] and its ligand hepatic growth factor (HGF) is associated with beta-cell differentiation [8-12]. C-met positive cells in the pancreas are considered to be multipotent and able to form cells of different pancreatic lineages. It was also shown that some c-met positive cells can be clonally expanded and therefore qualify as organ bound stem cell [5].

C-kit expressing cells in the pancreas are associated with neogenesis and reported to be expressed in the prenatal and postnatal rat pancreas, and during islet-cell development [1,6] and regeneration. Downregulation of c-kit results in decreased pdx-1 and insulin expression, suggesting downregulation of beta-cell differentiation [13].

Although nestin, c-met, and c-kit expression is associated with beta-cell development, it has never been shown that these proteins are expressed on beta-cells during differentiation or at which stage of beta-cells differentiation these proteins are expressed on beta-cells. We therefore first isolated nestin, c-kit and c-met positive cells from the neonatal pancreas and studied expression of transcription factors associated with pancreatic and beta-cell development in these cells. As the neonatal pancreas contains populations of beta-cells in different stages of development, we next studied the expression of nestin-, c-met, and c-kit on beta-cells in neonatal islets. Also, in a subsequent study we determined whether the expression was restricted to beta-cells or also occurs in differentiating pancreatic cells of non-beta-cell origin.

MATERIALS AND METHODS

Design of study
In order to decide upon the panel of transcription factors we should screen for on a protein levels we first applied a broad gene profile of nestin, c-met, and c-kit, positive cells isolated from the neonatal pancreas by RT-PCR assessments.
We quantified the mRNA levels of c-met, c-kit, nestin, Beta2 (transcription factor associated with beta-cell formation), Islet-1 (transcription factor specific for endocrine cell formation), Pax6 (transcription factor specific for alpha-cell formation), Pax4 (transcription factor specific for beta- and delta-cell formation), Pdx-1 (transcription factor specific for beta-cell formation), ngn3 (transcription factor specific for endocrine cell formation), Glut-2 (glucose transporter expressed by beta-cell), insulin (beta-cell secreted hormone), glucagon (alpha-cell secreted hormone), and amylase (acinar cell secreted enzyme). A selection of these proteins was applied in the subsequent immunofluorescent study in which we determined the expression of nestin, c-met, and c-kit on neonatal islets with beta-cells in either the early, mid-, or late stage of differentiation.

To study whether nestin, c-met, and c-kit cells is restricted to beta-cells or can also be found in other pancreatic cell types we studied the co-expression of nestin, c-met, and c-kit with the following markers; insulin or pdx-1 (beta-cells), glucagon (alpha-cells), pancreatic polypeptide (PP-cells) and somatostatin (delta-cells), CD31 (endothelial cells), and β3-tubulin (nerve cells).

**Animals**
All experiments were conducted in accordance with NIH-guidelines for the care and use of laboratory animals. Female Wistar rats (Harlan; age 3-4 months and weighing ~200 g) were kept in a temperature- and light-controlled room (lights on from 6 AM to 6 PM).

Animals to obtain pregnancy were subjected to daily vaginal smears. Pregnancy was achieved by housing the female rats on the night of pro-oestrus with a fertile male for one night. Rats were made pregnant and allowed to deliver naturally. Neonatal rats were used 1-2 days after delivery.

**Surgery**
The neonatal rat pancreata were obtained from 1-2 days old rat pups. The pups were decapitated and the pancreas was surgically removed. This was done by laparotomy, replacing the stomach aside, and taking the pancreas out by cutting it loose from the spleen, duodenum, and stomach wall. The neonatal pancreata were either snap frozen in liquid nitrogen and stored at -80°C until sectioning or
Nestin, c-met, and c-kit expression in differentiating neonatal beta-cells

processed for cell-isolation. For cell-isolation, the pancreata were stored in 5 ml of 0.20μm filtered Krebs-Ringer-Hepes buffer (KRH: 133 mM NaCl, 4.69 mM KCl, 1.18 mM KH$_2$PO$_4$, 1.18 mM MgSO$_4$·7H$_2$O, 10 mM HEPES and 2.52 mM CaCl$_2$·2H$_2$O (pH 7.4)) containing 5% bovine serum albumin (BSA) on ice.

Cell-isolation

Neonatal pancreata were digested with collagenase to transform them into single cells. The pancreata were first cut in small fragments of approximately 1 mm. The fragments were then washed once with KRH containing 5% BSA for 5 minutes at 4°C. The pancreas fragments were digested with 5.5 mg/ml Collagenase P (Boehringer Mannheim, Germany) in KRH containing 1% BSA for 12 minutes at 37°C under continuous agitation in a water bath. For the separation of the single cells from the debris, the mixture was centrifuged at 500 rpm for 30 seconds at 20°C. The supernatant (containing the single cells) was aspirated. The single cell suspension was washed 3 times with KRH containing 1% BSA for 5 minutes at 4°C. After washing, the cells were resuspended in KRH containing 1% BSA. Cell viability was determined with Trypan blue dye exclusion. The viability was always above 90%. After this procedure the single cells were divided in a portion for nestin, c-met, or c-kit cell-sorting.

According to standard methods the following procedure was performed for cell-sorting. First the single cells were centrifuged at 1500 rpm for 5 minutes. The supernatant was discarded and the pellet was pre-incubated with undiluted swine serum for 30 minutes. After incubation the cells were washed with PBS containing 0.5% BSA and 0.1% sodiumazide. Subsequently, the mixture was centrifuged at 1500 rpm for 5 minutes. Next the cells were incubated with the primary antibody anti-c-met (1:100) (H-190, Santa Cruz Biotechnology) or anti-c-kit (1:100) (C-19, Santa Cruz Biotechnology) for 60 minutes. Then the cells were incubated with secondary antibody swine-anti-rabbit FITC (1:50) (DakoCytomation, Denmark) for 30 minutes in the dark. After incubation the cells were washed with PBS for 5 minutes and resuspended in PBS. For cell-sorting of nestin positive cells we had to perform a different procedure since nestin is an intracellular protein. Therefore the single cell suspension was first permeabilised with PBS containing 0.5% saponin for 15 minutes and subsequently washed with PBS for 5 minutes. The cells were then incubated with primary antibody nestin (1:200) (Clone 401,
BD Biosciences), for 60 minutes. After incubation the cells were washed with PBS for 5 minutes. Finally, the cells were incubated with goat-anti-mouse-FITC (1:50) (Southern Biotechnology Associates, Inc). After incubation the cells were washed with PBS for 5 minutes and resuspended in PBS. The whole procedure was performed on ice. Positive selection for nestin, c-met and c-kit was performed using a Fluorescence Activated Cell Sorter (FACS, MoFlo flow cytometer) (Cytomation, USA). The sorted cells were collected in a tube with sterile RPMI 1640 containing 10% FCS, and 10 mg/ml Gentamycin (GIBCO). After cell sorting we examined the purity of the populations and we only included experiments where the purity of the populations were higher than 90%.

Transformation of isolated islets into single cells suspension
Islets of neonatal rat pancreata were isolated according to a collagenase digestion method as previously described. Briefly, islet isolation was performed in KRH containing 10% BSA. The pancreata were chopped in small fragments of 1 mm². Next the fragments were washed 3 times in KRH containing 10% BSA. Subsequently, the pancreas was digested using a two stage incubation of 20 min at 37°C with successively 1.0 and 0.7 mg/ml collagenase (Sigma type XI, Sigma, St Louis, MO, USA). Islets were separated from exocrine tissue by centrifugation over a discontinuous dextran gradient and further purified by handpicking. Islets were collected and washed in PBS twice before being dispersed into single cells by mechanical shaking at 37°C for 3 minutes in 0.05% Trypsin, 0.5 mM EDTA (Gibco). The enzymatic reaction was stopped by adding KRH 10% BSA. Cell viability was determined with Trypan blue dye exclusion indicated that more than 95% of the cells were viable after the procedure. Islets single cells were spotted on glass slides at a density of 50,000 cells per slide. This was done with a Cytospin centrifuge at 500 rpm for 5 min. Tissue cryosections were air dried and stored at -20°C until staining.

RNA isolation and cDNA synthesis
Sorted cells were washed with PBS. Total RNA was isolated using an Absolutely RNA® Minprep kit (Stratagene, Netherlands) according to the manufacturer’s protocol. Briefly, the cells were lysed in the lysisbuffer provided in the kit. Ethanol (70%) was added and the solution was placed in a RNA-Binding Spin Cup (containing a silica-based fiber matrix). The RNA binds to the silica-based fiber
Nestin, c-met, and c-kit expression in differentiating neonatal beta-cells

matrix. Possible genomic DNA contamination was removed by incubation with RNase-Free DNase I and DNase Digestion Buffer (Stratagene). Several washing steps removed contaminants and the RNA was collected using an elution buffer. RNA concentration was measured using The NanoDrop® ND-1000 Spectrophotometer.

The cDNA was synthesized from total RNA using SuperScript III reverse transcriptase (Invitrogen), RNaseOUT Recombinant Ribonuclease Inhibitor (Invitrogen), 0.1M DTT (Invitrogen), 5x First Strand Buffer (Invitrogen), dNTPs and random hexamer primers (Invitrogen) according to manufacturer’s protocol.

*Polymerase Chain Reaction analysis*

PCR reactions were performed in a MJ Research PTC-100 Thermal Cycler. The PCR reaction (total 30 μl) was performed as follows: 100 ng cDNA, 3 μl 10x PCR buffer (Fermentas), 3 μl dNTP’s (2mM, Fermentas), 1.25 μl MgCl₂ (50mM, Fermentas), 0.2 μl Taq DNA Polymerase (Invitrogen), 1 μl forward primer (40 mM), 1 μl reverse primer (40mM) and MilliQ water add up to 30 μl.

PCR cycles were as follows: denaturation of the double-stranded DNA at 94°C for 5 minutes, followed by 94°C for 30 seconds, primer annealing temperature for 30 seconds, 72°C for 30 seconds (40 cycles), and final extension at 72°C for 5 minutes. The PCR products were separated on a 2% agarose gel in 0.5x TBE running buffer (150V, 60 minutes), and visualized by ethidium bromide staining. The primer sequences, annealing temperatures, and the expected fragment sizes are shown in Table 1. β-Actin was used as a housekeeping gene for the PCR reaction.

*Immunofluorescence on sections of neonatal pancreata*

Neonatal pancreata were sectioned at 4 μm and then fixed in acetone for 10 minutes, followed by air drying for 30 minutes. The whole procedure was performed at room temperature.

For double staining of islet-1, ngn3, or amylase with c-met or c-kit the sections were treated as follows. The sections were incubated with normal donkey serum for 30 minutes and subsequently blocked with a biotin blocking system kit (DakoCytomation, Carpinteria, CA) for 15 minutes. Then the sections were incubated with primary antibody anti-islet-1(1:400) (K-20, Santa Cruz Biotechnology) anti-
ngn3 (1:400) (A-19, Santa Cruz Biotechnology), anti-amylase (1:400) (C-20, Santa Cruz Biotechnology) for 60 minutes; followed by secondary antibody Donkey-anti-goat-FITC (1:100) (AP180F, Chemicon International). Subsequently, the sections were incubated with normal goat serum for 30 minutes. Next the sections were incubated with primary antibody anti-c-met (1:150) or anti-c-kit (1:100) for 60 minutes. Then the sections were incubated with secondary antibody goat-anti-rabbit biotin (1:50) (DakoCytomation, Denmark) followed by tertiary antibody streptavidin-Cy3 (1:200) (Zymed laboratories, Inc) incubation for 30 minutes.

For double staining of islet-1, ngn3, or amylase with nestin the sections were treated as follows. The sections were incubated with normal donkey serum for 30 minutes. Then the sections were incubated with primary antibody anti-nestin (1:100) for 60 minutes; followed by secondary antibody Donkey-anti-mouse-TRITC (1:100) (AP192R, Chemicon International). Subsequently, the sections were incubated with normal donkey serum for 30 minutes. Next the sections were incubated with primary antibody anti-islet-1 (1:400) anti-ngn3 (1:400), anti-amylase (1:400) for 60 minutes. Finally, the sections were incubated with secondary antibody Donkey-anti-goat-FITC (1:100).

For double staining of Pax6 with c-met or c-kit the sections were treated as follows. The spots were incubated with normal goat serum for 30 minutes and subsequently blocked with a biotin blocking system for 15 minutes. Next the sections were incubated with primary antibody anti-Pax6 (1:400) (sc-53108, Santa Cruz Biotechnology) for 60 minutes. After that the sections were incubated with secondary antibody goat-anti-rabbit biotin (1:50) followed by tertiary antibody streptavidin-Cy3 (1:200) incubation for 30 minutes. Subsequently, the sections were incubated with primary antibody anti-c-met (1:150) or anti-c-kit (1:100) for 60 minutes. Next the spots were incubated with secondary antibody swine-anti-rabbit (FITC) (1:50) for 30 minutes followed by tertiary antibody goat-anti-swine (FITC) (1:50) (Jackson ImmunoResearch Laboratories, Inc) incubation for 30 minutes. Finally the sections were incubated with 4′, 6-diamidino-2-phenylindole (DAPI) (1:2500) for 10 minutes and mounted with Citifluor. Due to technical reasons, c-met and c-kit could not be doubled stained for Pax4.
Nestin, c-met, and c-kit expression in differentiating neonatal beta-cells

For double staining of Pax4 or Pax6 with nestin the sections were treated as follows. The sections were incubated with normal donkey serum for 30 minutes. Then the sections were incubated with primary antibody anti-nestin (1:100); followed by secondary antibody donkey-anti-mouse-FITC (1:100) or donkey-anti-mouse-TRITC (1:100). Subsequently, the sections were incubated with normal goat serum for 30 minutes blocked with a biotin blocking system kit for 15 minutes. Next the sections were incubated with primary antibody anti-Pax4 (1:150) (M-20, Santa Cruz Biotechnology) or anti-Pax6 (1:400) (sc-53108, Santa Cruz Biotechnology) for 60 minutes. After that the sections were incubated with secondary antibody goat-anti-rabbit biotin (1:50) followed by tertiary antibody streptavidin-FITC (1:100) or streptavidin-Cy3 (1:200) incubation for 30 minutes. Finally the sections were incubated with 4',6-diamidino-2-phenylindole (DAPI) (1:2500) (Roche) for 10 minutes and mounted with Citifluor (Agar Scientific). Analysis was performed using the Leica DMRXA fluorescent microscope and Leica Qwin Pro software.

**Immunofluorescence on cytopsots of islet cells**

Cytopsots were air dried and then fixed in acetone for 10 minutes, followed by air drying for 30 minutes. The whole procedure was performed at room temperature.

For double staining of c-met or c-kit with insulin or glucagon and Polypeptide and Somatostatin with nestin the spots were treated as follows. The spots were incubated with normal goat serum for 30 minutes and subsequently blocked with a biotin blocking system kit for 15 minutes. Subsequently, the sections were incubated with primary antibody anti-c-met (1:150) or anti-c-kit (1:100) or anti-PP (Abcam) 1:600, anti-Somatostatin (Sigma Aldrich) 1:800) for 60 minutes. Next the spots were incubated with secondary antibody goat-anti-rabbit biotin (1:50) followed by tertiary antibody streptavidin-Cy3 (1:200) incubation for 30 minutes. Then the spots were incubated with primary antibody anti-insulin (1:750) (Sigma-Aldrich, The Netherlands), or anti-glucagon (1:2000) (Sigma-Aldrich, The Netherlands), or anti-nestin (1:500); followed by secondary antibody goat-anti-mouse-FITC (1:50). Since the antibodies of c-met, c-kit and Polypeptide and Somatostatin are of the same isotype we could not perform the double staining for these proteins. Also the antibodies of nestin and glucagon are of the same isotype therefore we could not perform the double staining for these two proteins. In addition, the double staining of nestin and insulin could also not be done, because the antibodies are of the same
isotype. Since it is important for the line of reasoning (vide infra) to determine whether nestin positive cells have feature of beta-cells we decided to perform a staining for nestin with Pdx-1. For double staining of Pdx-1 or β3-tubulin with nestin the spots were treated as follow: the spots were incubated with normal goat serum for 30 minutes at room temperature and subsequently blocked with the biotin blocking system kit for 15 minutes. Subsequently, the spots were incubated with primary antibody anti-Pdx-1 (P9995, Sigma Aldrich Inc.,) (1:100), or anti-β3-tubulin (gift from Dr. W. Baron) for 60 minutes. Next the spots were incubated with secondary antibody goat-anti-rabbit biotin (1:50) followed by tertiary antibody streptavidin-Cy3 (1:200) or goat-anti-mouse-tetramethyl rhodamine iso-thiocyanate (TRITC) (1:50) (DakoCytomation, Denmark) incubation for 30 minutes. Then the spots were incubated with primary antibody anti-nestin (1:100); followed by secondary antibody goat-anti-mouse-FITC (1:50). The whole procedure was performed at room temperature.

For double staining of c-met or c-kit with β3-tubulin the spots were treated as follows. The spots were incubated with normal goat serum for 30 minutes and subsequently blocked with the biotin blocking system kit for 15 minutes. Subsequently, the spots were incubated with primary antibody anti-c-met (1:150) or anti-c-kit (1:100) for 60 minutes. Next the spots were incubated with secondary antibody goat-anti-rabbit biotin (1:50) followed by tertiary antibody streptavidin-Cy3 (1:200) or goat-anti-mouse-FITC (1:50) incubation for 30 minutes.

For double staining of nestin, c-met, or c-kit with CD31 we followed the following procedure. The spots were incubated with normal goat serum for 30 minutes and subsequently blocked with a biotin blocking system for 15 minutes. Subsequently, the spots were incubated with primary antibody anti-nestin (1:100) or anti-c-met (1:150) or anti-c-kit (1:100) for 60 minutes. Next the spots were incubated with secondary antibody goat-anti-mouse IgG1 fluorescein isothiocyanate FITC (1:50) (for nestin) or goat-anti-rabbit biotin (1:50) (for c-met) or swine-anti-rabbit (FITC) (1:50) (for c-kit) for 30 minutes followed by tertiary antibody streptavidin-Cy3 (1:200) or goat-anti-swine (FITC) (1:50) (Jackson ImmunoResearch Laboratories, Inc) incubation for 30 minutes. Then the spots were incubated with primary antibody anti-CD31-
Nestin, c-met, and c-kit expression in differentiating neonatal beta-cells

biotinylated (1:50) for 60 minutes. Subsequently, the spots were incubated with secondary antibody streptavidin-Cy3 (1:200) for 30 minutes. Finally the spots were incubated with 4’, 6-diamidino-2-phenylindole (DAPI) (1:2500) for 10 minutes and mounted with Citifluor. Analysis was performed using the Leica DMRXA fluorescent microscope equipped with Leica DC 350 FX and Leica Qwin Pro software.

RESULTS

Although it has been shown that cells expressing nestin, c-met, and c-kit expression are essential for normal development of the pancreas, minor knowledge is available about the frequency of the cells expressing these proteins, the expression of transcription factor associated with development of the pancreas in these cells, and to which cell-types these cells will differentiate in vivo. We started out by isolating pure and fresh populations of these cells from the neonatal pancreas, which allowed us the study the expression of mRNA of various transcription factors associated with pancreatic development.

After transforming neonatal pancreata into single cells, we found that 25.0 ± 1.7% of the cell population are nestin positive, 1.8 ± 0.9% are c-met positive cells, and 4.7 ± 0.8% are c-kit positive cells (Table 1). These individual populations were purified by cell sorting. Because it is difficult to isolate sufficient amounts of RNA from permeabilised intracellularly stained nestin positive cells, we could only quantify a limited number of genes. As nestin has been mostly described as a protein associated with endocrine cells we choose the transcription factors ngn3, Pax4, and Pdx-1. We found that nestin positive cells expressed ngn3, but not Pax4, or Pdx-1 (Figure 1A). The sorted c-met or c-kit positive cells were subjected to a broader analysis, since we could obtain more RNA from these cells after cell-sorting. We quantified the mRNA levels of c-met, c-kit, nestin, Beta2, Islet-1, Pax6, Pax4, Pdx-1, ngn3, Glut-2, insulin, glucagon, and amylase. C-met sorted cells express c-met, c-kit, nestin, Beta2, Pax6, ngn3, Pdx-1, insulin, glucagon, and amylase on mRNA levels (Figure 1B). C-kit sorted cells express c-kit, Beta2, Islet-1, Pax4, insulin, and amylase on mRNA levels (Figure 1C).
Table 1: Percentage of nestin, c-met, and c-kit positive cells in the neonatal pancreas.

<table>
<thead>
<tr>
<th>Cells in the neonatal pancreas</th>
<th>Percentage of positive cells</th>
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<tbody>
<tr>
<td>Nestin positive cells</td>
<td>25.0 ± 1.7</td>
</tr>
<tr>
<td>C-met positive cells</td>
<td>1.8 ± 0.9</td>
</tr>
<tr>
<td>C-kit positive cells</td>
<td>4.7 ± 0.8</td>
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Transcription factors in the endocrine and exocrine tissues

From the above follows that c-met, c-kit and nestin positive cells express various transcription factors associated with beta-cell development. By sorting cells from the whole pancreas, we sorted a heterogeneous population of c-kit, c-met, and nestin positive cells, since these cells can be found not only in islets but also around the ducts and vasculature as well as in exocrine tissue (chapter 4). Therefore, we next studied in histological sections which of the cell populations expressed the beta-cell specific transcription factors.

We performed double staining with an early, mid and late markers for endocrine cell differentiation and an exocrine marker. We define islet-1 as an early-stage protein for endocrine differentiation [14], ngn3 as a mid-stage protein for endocrine differentiation [14], and Pax4 or Pax6 as late-stage proteins for beta-cell differentiation and delta-cell differentiation [14]. We studied in nestin positive cells islet-1, ngn3, Pax4, or Pax6, and in c-met and c-kit positive cells islet-1, ngn3, and Pax6, on a protein level. Due to technical reasons (see materials and methods), c-met and c-kit could not be double stained for Pax4. We furthermore investigated whether nestin, c-met, and c-kit cells also expressed the late exocrine marker amylase [15].

Nestin positive cells were abundantly present in both the exocrine and endocrine pancreas. The nestin positive cells in the endocrine pancreas, *i.e.* the islets, co-express islet-1 (Figure 2A) and ngn3 (Figure 2B), the early and late stage markers for beta-cells. We furthermore found that nestin positive cells do not co-express Pax4 (Figure 2C) or Pax6 (Figure 2D), *i.e.* transcription factors associated with beta-cell and alpha-cell formation. Surprisingly, although we found nestin positive cells in the exocrine pancreas, the cells never expressed amylase (Figure 2E).
Figure 1: Agarose gel electroforesis of RT-PCR products of freshly isolated nestin, c-met, and c-kit positive cells. Nestin, c-met, and c-kit positive cells were isolated from neonatal pancreata and following RNA isolation and cDNA synthesis subjected to RT-PCR. Nestin positive cells were checked for the gene transcripts of Pax4, Ngn3 and Pdx-1; and c-met and c-kit positive cells were checked for the gene transcripts of c-met, c-kit, nestin, Beta2, Islet-1, Pax6, Pax4, Pdx-1, Ngn3, Glut-2, insulin, glucagon, and amylase. B-actin was used as a housekeeping gene. Note that nestin positive cells express Ngn3 and not the late genes for Pax4 or Pdx-1 (A). C-met positive cells express c-met, c-kit, nestin, Pax6, Pdx-1, insulin, glucagon, and amylase, but not Islet-1, Pax4, Glut-2 on mRNA levels (B). C-kit positive cells express c-kit, Pax4, insulin, and amylase, but no c-met, nestin, Pax6 Ngn3, Pdx-1, Glut-2 or glucagon on mRNA levels (C).

C-met positive cells were found as scattered spots throughout both the endocrine and exocrine pancreas. C-met is clearly expressed in the whole period of beta-cell differentiation as it was found on cells expressing islet-1 (Figure 3A), ngn-3 (Figure 3B) and Pax-6 (Figure 3C). Notably we found that c-met positive cells were negative for islet-1 mRNA. C-met expression was also found on amylase producing exocrine cells (Figure 3D).
Figure 2: Photomicrograph of the neonatal rat pancreas after immunostaining for nestin with the early, mid and late markers for endocrine cell differentiation and the late exocrine marker. The sections were immunostained for nestin with Islet-1, nestin with ngn3, nestin with Pax4, nestin with Pax6, and nestin with amylase. Nestin positive cells in the islets of the neonatal pancreas co-express islet-1 (A) and ngn3 (B), but not Pax4 (C), Pax6 (D) or amylase (E). Original magnification 200X.
Figure 3: Photomicrograph of the neonatal rat pancreas after immunostaining for c-met with the early, mid and late markers for endocrine cell differentiation and the late exocrine marker. The sections were immunostained for c-met with Isl-1, c-met with ngn3, c-met with Pax4, c-met with Pax6, and c-met with amylase (E). C-met positive cells in the islets of the neonatal pancreas co-express Isl-1 (A), ngn3 (B), Pax4 (C), Pax6 (D) and amylase (E). Original magnification 200X
Figure 4: Photomicrograph of the neonatal rat pancreas after immunostaining for c-kit with the early, mid and late markers for endocrine cell differentiation and the late exocrine marker. The sections were immunostained for c-kit with Islet-1, c-kit with ngn3, c-kit with Pax4, c-kit with Pax6, and c-kit with amylase (E). C-kit positive cells in the islets of the neonatal pancreas co-express islet-1 (A), ngn3 (B), Pax4 (C), Pax6 (D) and amylase (E). Original magnification 200X.
Also c-kit expression can be found in all stages of beta-cell differentiation. C-kit was found on cells expressing islet-1 (Figure 4A), ngn-3 (Figure 4B) and Pax-6 (Figure 4C). Similar to c-met we also found c-kit on amylase producing exocrine cells (Figure 4D).

**Co-expression with other pancreatic cell types**

From the foregoing follows that nestin, c-met, and c-kit positive cells are clearly expressed by differentiating beta-cells in the islets. A striking observation is that almost all differentiating beta-cells express either nestin, c-met or c-kit, but that not all nestin, c-met, or c-kit expressing cells produce transcription factors associated with beta-cell differentiation. This implies that also other cell types in the neonatal islets do express nestin, c-met, or c-kit. Therefore, in a subsequent study we co-stained nestin, c-met or c-kit with either the beta-cell specific markers Pdx-1 or insulin; the alpha-cell specific marker glucagon; the PP-cell and delta-cell specific markers pancreatic-polypeptide and somatostatin; the endothelial cell specific marker CD31; and nerve cell specific marker β3-tubulin. This was done on purified population of islet cells to obtain a more detailed analysis on cell level.

We found that nestin positive cells do not express Pdx-1 (Figure 5A). A few nestin positive cells expressed pancreatic-polypeptide and somatostatin, but this was only a fraction of the pancreatic-polypeptide and somatostatin producing cells in the islets (Figure 5B). Another portion of the nestin expressing cells are CD31 positive endothelial cell (Figure 5C) and β3-tubulin expressing nerves in the islets (Figure 5D). Thus nestin is predominantly expressed on early differentiating beta-cells, on endothelial cells, and on nerve cells in the islets.

Also c-met expression is not restricted to beta-cells. Although these cells express the beta-cell specific marker insulin (Figure 6A), we also found c-met on glucagon (Figure 6B) producing alpha-cells. The fast majority of c-met positive cells were CD31 positive endothelial cells (Figure 6C) and β3-tubulin positive nerve cells (Figure 6D).

Similar to what we found on c-met positive cells, we found c-kit expressing cells (Figure 7A) and on glucagon producing cells (Figure 7B). However, the fast majority of c-kit bearing cells were CD31 positive endothelial cells (Figure 7C).
and β3-tubulin positive nerve cells (Figure 7D). Due to technical reasons (see materials and methods), c-met and c-kit could not be doubled stained for pancreatic-polypeptide and somatostatin.

Figure 5: Photomicrograph of cytopsots of single cells from neonatal rat islets after immunostaining for nestin and mature islet cells, endothelial cells, and nerve cells. The sections were immunostained for nestin with Pdx-1, nestin with polypeptide and somatostatin, nestin with CD31, and nestin with β3-tubulin. Nestin positive cells do not express Pdx-1 (A). A few nestin positive cells express pancreatic-polypeptide and somatostatin (B). Except for islet cells, nestin positive cells also co-express CD31 (C), and β3-tubulin (D). Original magnification 200X
Figure 6: Photomicrograph of cytospots of single cells from the neonatal rat islets after immunostaining for c-met and mature islet cells, endothelial cells, and nerve cells. The sections were immunostained for c-met with insulin, c-met with glucagon, c-met with CD31, and c-met with β3-tubulin. C-met positive cells express insulin (A) and glucagon (B). C-met positive cells also co-express CD31 (C), and β3-tubulin (D). Original magnification 200X
DISCUSSION

In the present study we show for the first time that nestin, c-met, and c-kit are differentially expressed by beta-cells during the process of differentiation. Nestin is expressed by beta-cells in the early and mid-stage of differentiation and is absent...
Nestin, c-met, and c-kit expression in differentiating neonatal beta-cells

on beta-cells at the late or end stage of differentiation. C-met and c-kit are receptors that are found on all stages of differentiation of beta-cells.

The fast majority of studies on the role of nestin, c-met, and c-kit associate these bioactive molecules with islet-cell development, regeneration or with engineering and clonal expansion of beta-cell precursors for generation of beta-cells [1,3,5,6]. Only a few studies have focussed on the expression of these molecules on other cell types in the pancreas. We show that nestin, c-met, and c-kit is not only found on differentiating beta-cells but also on the non-endocrine cells in the islets, i.e. differentiating neonatal blood vessels and nerves.

In 2001, Zulewski [3] demonstrated that nestin positive cells isolated from rat and human pancreatic islets can become beta-cells after appropriate stimulation in vitro. This however, was been debated by many who claimed that nestin positive cells were precursors for other cell types such as endothelial such but not for beta-cells [16-19]. Our results illustrate however that nestin positive cells express both Islet-1 and ngn3 which have been previously been described as key regulators for pancreas development [20,21]. Our results corroborate the findings of Zulewski [3] but do not necessarily debate the findings of the other groups [16-19] since we also show that in the pancreatic islets nestin can be found not only in beta-cells but also in endothelial cells and in nerve cells. This implies that also in clonal expansion studies researchers may end up with nestin positive precursors that are not beta-cell precursors.

Interestingly also recent papers on human pancreata support our findings and that of Zulewski on nestin in precursors of beta-cells. Eberhardt shows that outgrowing nestin positive cells from human islets express Islet-1 [22]. Furthermore it has been reported that in human foetal pancreas nestin positive cells co-express ngn3 in ducts, but not in the islets [23]. This is different in the postnatal rat pancreas since we found that nestin positive cells co-express ngn3 in the islets.

The presence of progenitor cells in the pancreas has been questioned over the past few years. It has been thought that newly formed beta-cells are the product of beta-cell replication [24], whereas others have pleaded for the existence of precursor cells and their contribution to newly formed beta-cells [25-31], however the existence
was not proven. This has recently changed by the pioneering publication of Xu et al. [32]. These authors demonstrate the presence of a multipotent progenitor cell in the stressed adult pancreas. Interestingly this multipotent progenitor cell is ngn3 positive just as the pancreas derived multipotent c-met cells that can be clonally expanded and can differentiate towards insulin positive cell when infused in the pancreas [5]. Also our c-met and nestin positive cells are ngn3 positive and show similar distribution patterns in the pancreas as the cells described by Xu et al. [32].

C-met expression is believed to be crucial in beta-cell development [8]. It has been reported that isolated c-met positive cells from the pancreas can be clonally expanded to become insulin, glucagon or amylase positive [5]. Our results suggest that this multipotency is only observed in vitro after ex vivo manipulation and that this does not normally occur in vivo.

C-kit is mainly expressed in the endocrine alpha- and beta-cell fraction since the c-kit positive islet cells express Islet-1, ngn3 but also Pax6 which is associated with alpha-cell development [33]. To our best knowledge, c-kit has not been associated with alpha-cell differentiation, before. Also new from our data is the fact that c-kit is expressed on CD31 expressing endothelial cells and β3-tubulin expressing nerve cells in the islets of the neonatal rat pancreas. This suggests that c-kit positive cells are not only involved in formation of endocrine cells, but also in formation of islet vasculature and innervation. It has been shown however that c-kit expression is essential for beta-cell development. Wang et al. [2] demonstrated that downregulation of c-kit results in decreased pdx-1 and insulin expression, suggesting downregulation of beta-cell differentiation [13].

Our study was not only undertaken to study the expression patterns of nestin, c-met, and c-kit cells in the differentiating endocrine pancreas, but also to confirm that cells expressing these molecules are indeed precursors for beta-cells. Many are the efforts to find ligands on precursor cells that allow selection of cells that have the potential to become beta-cell [4,5,34]. We demonstrate in this study that a portion of the nestin, c-met, and c-kit positive cells are indeed precursors for beta-cells. Therefore, a conceivable approach is to apply the extracellular proteins c-met and c-kit as a selection molecule for isolating precursor cells from the endocrine pancreas. Further purification before clonal expansion is applied may
Nestin, c-met, and c-kit expression in differentiating neonatal beta-cells occur via deletion of the c-kit and c-met expressing endothelial cells and nerve cells.

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REFERENCE LIST


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Shanti T. Gangaram-Panday, MSc, Marijke M. Faas, PhD and Paul de Vos, PhD

University Medical Center Groningen, University of Groningen, Dep. Pathology and Medical Biology, Div. of Medical Biology, Immunoendocrinology, Hanzeplein 1, 9700 RB Groningen, The Netherlands.